Influence of Autoinducer-2 (AI-2) and Beef Sample Extracts on *E. coli* O157:H7 Survival and Gene Expression of Virulence Genes *yadK* and *hhA*

K.A. Soni, L. Lu, P.R. Jesudhasan, M.E. Hume, and S.D. Pillai

ABSTRACT: Bacterial cell-to-cell communication is mediated by autoinducer (AI) molecules such as AI-2 and has been reported to regulate gene expression in *Escherichia coli* O157:H7. We have previously shown that ground beef contains compounds that can inhibit sensing of AI-2 like activity. The hypothesis of this study was that AI-2 activity observed in conditioned medium (CM) will enhance *E. coli* O157:H7 survival and expression of virulence genes, whereas compounds inhibitory (such as those present in ground beef extracts) to AI-2 activity will negate these effects. *E. coli* O157:H7 *luxS* mutant strain VS 94 (incapable of synthesizing AI-2) was employed in these studies. The survival of this enteric bacterial pathogen as a function of AI-2 activity and the presence of AI-2 inhibitory compounds was studied at 4 °C. The number of survivors in the presence of AI-2 was significantly higher compared to the absence of AI-2, and the addition of ground beef extracts to conditioned medium negated the influence of AI-2 activity. Autoinducer AI-2 upregulated selected genes virulence genes (*yadK*, and *hha*), whereas the ground beef extract reversed the effect of AI-2 on the expression of the selected genes.

Keywords: AI-2 molecules, autoinducers, *E. coli* O157: H7, inhibitory compounds

Introduction

Studies have shown that a variety of microbial processes such as growth, sporulation, toxin production, virulence, antibiotic synthesis, and motility in bacterial cells are coordinately regulated at the gene expression level by a variety of intra-and intercellular autoinducer molecules in a process termed quorum sensing (QS) (Sperandio and others 2003; Xavier and Bassler 2003; Pillai and Jesudhasan 2007). Currently, 4 different autoinducer (AI) molecules have been reported in the literature, namely, AI-1 (Bassler and others 1997), AI-2 (Schauder and others 2001), AI-3 (Sperandio and others 2003), and AI-peptides (Morrison 1997). Among the different AI molecules, AI-2 has been considered the universal signaling molecule since the *luxS* gene, which is involved in the production of AI-2, is widely conserved among the different bacterial species, including *Escherichia coli* (Xavier and Bassler 2003).

E. coli O157:H7 is a widespread infectious human pathogen responsible for a high incidence of gastrointestinal illnesses, including hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HS). Food animals are the most important source of human infections related to *E. coli* O157:H7. Both diseased and healthy ruminants, especially cattle, are main reservoirs of this pathogen (Todd and Dundas 2001; Li and Hovde 2007). Of the entire *E. coli* genome, about 5% to 10% of the genes are controlled by AI-2 molecules present in the conditioned medium (CM) (DeLisa and others 2001; Sperandio and others 2001).

MS 20070642 Submitted 8/21/2007, Accepted 10/31/2007. *Authors Soni, Jesudhasan, and Pillai are with Dept. of Poultry Science & Nutrition and Food Science, Texas A & M Univ., College Station, TX 77843, U.S.A. Author Lu is currently at Yale Univ. School of Medicine, New Haven, CT, U.S.A. Author Hume is with Food and Feed Safety Research Unit, USDA-ARS, College Station, TX, U.S.A. Direct inquiries to author Pillai (E-mail: spillai@poultry.tamu.edu).*

Over the last decade, a significant amount of work has been published on quorum sensing at the molecular level (Waters and Bassler 2005; Walters and Sperandio 2006). However, only recently has its importance in foods been recognized (Bruhn and others 2004; Lu and others 2004, 2005; Widmer and others 2007). Foods contain a multitude of ingredients that can potentially repress or enhance cell–cell communication, so the ultimate survival or activity of a particular pathogen or spoilage organism can be very different in different foods (Pillai and Jesudhasan 2007). We had previously shown that uncooked ground beef contains compounds that interfere with AI-2 activity (Lu and others 2004). Here, we hypothesized that the presence of AI-2 molecules will have protective effects on the survival of *E. coli* O157:H7 and influence its virulence gene expression. Furthermore, since ground beef extracts have been shown to inhibit the expression of AI-2 activity, we hypothesized that the introduction of ground beef extracts with AI-2 molecules will negate the influence of AI-2 molecules.

Material and Methods

Preparation of the ground beef extracts

To prepare the ground beef extracts, ground beef patties (approximately 15% fat content) purchased from a commercial source were mixed (1:1, w/v) with 0.1 M phosphate buffer (PB) in plastic bags, followed by stomaching for 2 min. After stomaching, the preparation was centrifuged (4000 × *g*) and filtered-sterilized using a 0.2 μm filter.

Bacterial strains

E. coli O157:H7 *luxS* mutant strain VS 94 (*luxS*$^-$) that cannot produce AI-2 but can respond to exogenously supplied AI-2 molecules (Sperandio and others 2001) was used in this study. The VS 94

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strain was a kind gift provided by Dr. Sperandio (UT Southwestern Medical School). *E. coli* O157:H7 strain ATCC 43895 (*luxS*$^+$) was used to prepare condition medium (CM) containing AI-2-like activity. Luria–Bertani (LB) broth supplemented with 0.5% of glucose was used to culture *E. coli* O157:H7 at 37 °C. The reporter strains *Vibrio harveyi* BB170 (*luxN*::Tn5 sensor 1$^-$ sensor 2$^+$) were used in the AI-2 activity bioassay as described previously (Lu and others 2004). *V. harveyi* BB170 cells were grown in the autoinducer bioassay (AB) medium at 30 °C with shaking and aeration (Surette and Bassler 1998).

Preparation of conditioned medium (CM) containing AI-2 activity

An overnight culture of ATCC 43895 was inoculated in LB broth (1:100) supplemented with 0.5% glucose and grown to an OD_{600} of 1.2. The supernatant was collected by centrifugation at 6000 × *g* for 15 min, passed through 0.22-μm filters, and resultant preparation was termed as conditioned medium (CM). Conditioned medium exhibited AI-2 activity as determined by the *V. harveyi* bioassay (Lu and others 2004). Heat treatment has been previously shown to destroy this autoinducer (AI-2) activity (Surette and Bassler 1998). A portion of the CM was heat inactivated using autoclaving conditions (121 °C, 15 psi, and 15 min) and this preparation was termed as autoclaved conditioned medium (ACM). AI-2 activity in both the CM and ACM was checked using AI-2 activity bioassay. The AI-2 activity observed in the CM preparations was also checked for its stability over 20 d at 4 °C.

AI-2 activity bioassay

V. harveyi reporter strains BB170, which exhibit bioluminescence in the presence of AI-2 autoinducer molecules, were used to confirm the autoinducer activity in CM (Lu and others 2004). Briefly, 90 μL of the freshly diluted (1:5000) cultures of reporter strain in autoinducer bioassay (AB) medium were mixed with 10 μL samples. In addition, negative control (10 μL of AB media) was placed for each experiment. Samples in the plate were placed at 30 °C with moderate shaking of 100 RPM and luminescence responses of the reporter strains were measured at regular time interval using a Wallac 1420 plate reader (PerkinElmer, Shelton, Conn., U.S.A.) until negative control started showing luminescence.

Inhibition of AI-2 activity

The objective of these experiments was to verify that ground beef extract is able to inhibit the AI-2 activity. The experimental approach to determine inhibition of AI-2 activity by ground extracts was adapted from previous report (Lu and others 2004). Briefly, 90 μL of the freshly diluted (1:5000) cultures of reporter strain in autoinducer bioassay (AB) medium were mixed with 5 μL of CM + 5 μL of ground beef extracts in a 96-well plate. In addition, negative control (10 μL of AB media) and positive control (5 μL of CM + 5 μL of AB media) were placed for each experiment. Plates were placed at 30 °C and luminescence responses of the reporter strains were measured. Inhibition of AI-2 activity was expressed in relation to the positive control. The bioluminescence observed in the positive control was considered to be 100%.

Survival of *E. coli* O157:H7 in the presence of AI-2

The influence of AI-2 like activity on the survival of *E. coli* O157:H7 VS 94 cells was studied by monitoring the survival in CM, ACM, and phosphate buffer (PB). The CM was used as a source of AI-2 activity, and ACM and PB were used as controls. Aliquots of the washed *E. coli* cells were suspended in sterile borosilicate glass tubes with either 10 mL of CM or ACM or PB to yield a final cell suspension of approximately 10^4 CFU/mL. The cell suspensions were incubated at 4 °C for 30 d and at periodic intervals (0, 5, 10, 15, and 20 d) the viable cell numbers (CFU/mL) were estimated by diluting in phosphate buffer (0.1 M) and plating on LB agar. The plates were incubated at 37 °C for 24 h prior to enumeration.

Survival of *E. coli* O157:H7 in the presence of AI-2 and ground beef extracts

The objective of this experiment was to determine if the presence of ground beef extracts negated the influence of AI-2 molecules on the survival of *E. coli* O157:H7. Portions (5 mL) of ground beef extracts were added to 5 mL of CM, 5 mL of autoclaved ACM, and 5 mL of PB (1:1 ratio) in sterile glass tubes. The washed *E. coli* O157:H7 VS94 cells were inoculated into the different beef extract treatments at a final concentration of approximately 10^4 CFU/mL. The samples were incubated at 4 °C for 30 d. At periodic time intervals, aliquots were removed and plated on LB agar. The colonies were enumerated after 24 h incubation at 37 °C.

Influence of AI-2 and ground beef extracts on virulence gene expression

Overnight culture of *E. coli* O157:H7 (VS 94) was inoculated separately (1% [vol/vol] inoculum) in fresh LB broth supplemented with 0.5% of glucose and grown aerobically to 1.0 OD_{600nm}. Once an $OD_{600nm} = 1$ was reached, the cells were divided into 3 mL aliquots in separate tubes and centrifuged for 5 min (3500 × *g* at 4 °C). At this time point, the supernatant was completely removed and cell pellets were resupended with CM, ACM, or CM and ACM mixed with ground beef extracts in equal proportion. The resuspended cells were incubated for 25 min of aerobic growth conditions in a shaking incubator (37 °C). The cell suspensions were centrifuged for 5 min (3500 × *g* at 4 °C) and the bacterial pellets were saved. Total RNA from the cell pellets was extracted using the commercial RNA extraction kit (Ribopure $^{TM-}$ Bacteria; Ambion Inc., Austin, Tex., U.S.A.). The extracted RNA was stored at –80 °C until used for cDNA synthesis.

The cDNA synthesis reagents (Gene Amp® RNA PCR Kit) were obtained from Applied Biosystems (Branchburg, N.J., U.S.A.). The master mix preparation was prepared as mentioned in the instruction manual. The 1st strand synthesis of cDNA was carried out using single cycle (60 min at 42 °C for annealing and elongation followed by 5 min at 99 °C to inactivate the reverse transcriptase enzyme) reverse-transcriptase polymerase chain reaction (RT-PCR). The RT- PCR cycle conditions were as follows.

The cDNA was used as template in real-time PCR analysis. The primer sequences that were employed are shown in Table 1. The bacterial 16S rRNA gene was used for the normalization of the gene expression data. The 384-well clear optical reaction plate (Applied Biosystems) was filled with 1 μL of cDNA (template) and 19 μL of the master mix. The master mix placed in individual wells consisted of 10 μL of SYBR® GREEN PCR mix (Applied Biosystems,

Table 1—Primer sequence for selected genes used in gene expression studies.

Gene[a]	Primer sequence
hha-F	5′-ATAATGAACTGGCGGTATTTTACTCA-3′
hha-R	5′- GTCGTACAGTTTATTCATGGTCAATTC-3′
yadK-F	5′- AACGTCGGCATTGTGATTTT-3′
yadK-R	5′- TCCGTTCCTCGCAGTTAA-3′
16S-F	5′- CCAGCAGCCGCGGTAAT-3′
16S-R	5′- TGCGCTTTACGCCCAGTAAT-3′

[a]F = forward primer, R = reverse primer.

Warrington, U.K.), primers (0.6 μL [10 μmol] for *hha*-F, *hha*-R, *yadK*-F, and *yadK*-R; 0.9 μL [10 μmol] for *16S*-F and *16S*-R) and volume was adjusted to 19 μL using DEPC-treated deionized water. The instrument was programmed for relative quantification to obtain a C_T value and dissociation curve. The amount of primer added in the master mix was based on preliminary primer optimization trials.

Statistical analysis

All experiments were performed in triplicate (3 biological replicates and 3 technical replicates for each biological replicate). Significant differences, if any, in various experimental treatments were analyzed using a paired *t*-test or 1-way analysis of variance (ANOVA) by SPSS version 12.0 (Chicago, Ill., U.S.A.). The changes in gene expression were calculated as either fold-increase or fold-decrease using the mathematical formula: treatment 2/treatment $1 = 2^{\Delta\Delta C}{}_T$, where $\Delta\Delta C_T = \Delta C_T$ for treatment 1 – ΔC_T for treatment 2 and ΔC_T is the difference between the C_T value of the target gene and the normalization gene (16S).

Results and Discussion

Stability of AI-2 activity at 4 °C

Prepared CM was stored at 4 °C and tested at periodic time intervals (0, 5, 10, 15, and 20 d) to check its ability to induce luminescence response (measure of AI-2 activity) in *V. harveyi* BB170 reporter strain. The level of light induction in *V. harveyi* BB170 reporter strain was the same during all time points, ensuring that AI-2 molecules were stable at refrigeration temperature (data not shown). When ACM (heat inactivated CM) was used as a sample, no induction of luminescence was observed and this observation was consistent with a previous report (Surette and Bassler 1998).

Inhibition of AI-2 activity by ground beef extracts

The ability of ground beef extracts to inhibit expression of AI-2 activity based bioluminescence was tested as previously described by (Lu and others 2004). When tested, the presence of ground beef extracts resulted in >90% inhibition of AI-2 controlled bioluminescence in *V. harveyi* bio-assays (Figure 1). These results were in agreement with previous reports where similar level of AI-2 inhibition was observed (Lu and others 2004).

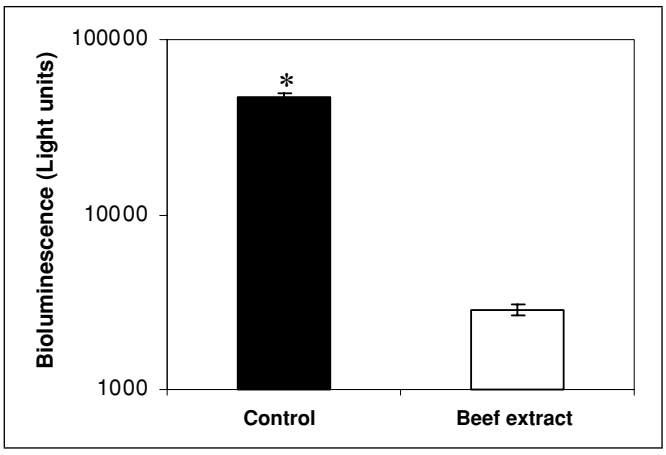

Figure 1—Inhibition of AI-2 induced bioluminescence by ground beef extracts. Ability of ground beef extracts to inhibit bioluminescence expression was determined using *V. harveyi* BB170 reporter strain based autoinducer bioassay. *Represents statistical significant difference ($P \leq 0.05$) based on paired *t*-test.

AI-2 influences survival of *E. coli* O157:H7 VS94 strain

There were marked differences in the survival of *E. coli* O157:H7 (VS94) cells in the presence of CM, ACM, and PB (Figure 2). Higher number of bacterial cells survived in the presence of CM (contains AI-2 molecules) than ACM and PB after 10 d; however, there were no significant differences observed at the end of 5 d. At the end of 20 d more than 50% of the initial cell number survived in the presence of CM compared to 19% (±5.2) and 5% (±4.8) in the presence of ACM and PB, respectively. There were no significant differences between ACM and PB during any time points.

Ground beef extracts negates the influence of AI-2 molecules on the survival of *E. coli* O157:H7 VS94 strain

In the presence of ground beef extracts that contained demonstrable levels of compounds inhibitory to AI-2 activity, *E. coli* O157:H7 cells declined in numbers irrespective of whether AI-2 molecules were extraneously added in the form of CM (Figure 3).

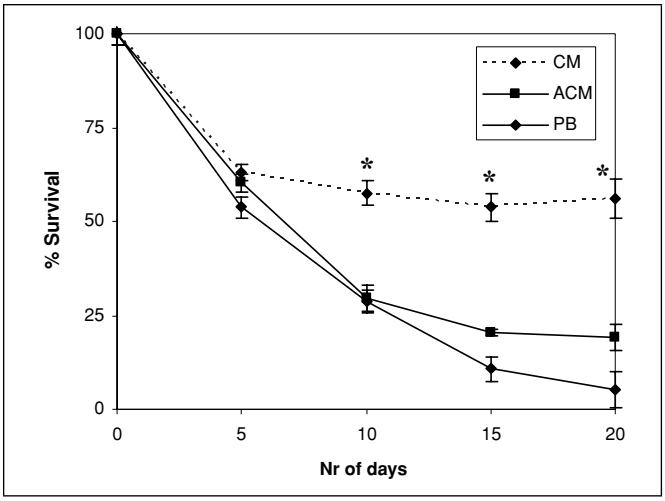

Figure 2—Percentage survival of *E. coli* O157:H7 (VS94) strain in the presence of AI-2 (conditioned medium [CM]) and its absence (autoclaved conditioned medium [ACM] and phosphate buffer [PB]). *Represents statistical significant difference ($P \leq 0.05$) based on paired *t*-test.

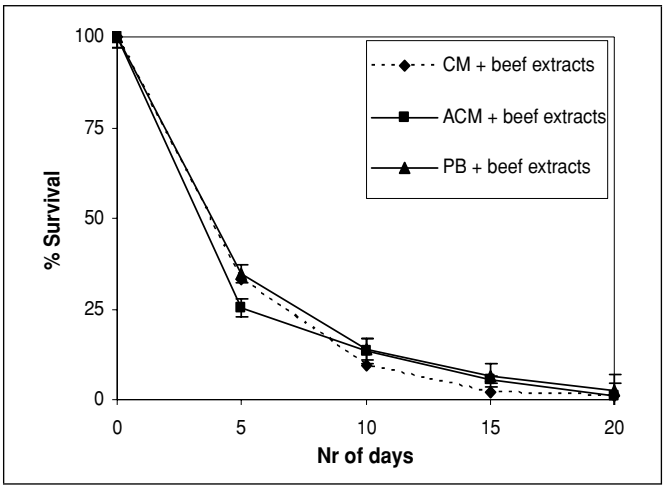

Figure 3—Percentage survival of *E. coli* O157:H7 (VS94) strain in the presence of beef extract amended AI-2 (conditioned medium [CM]) or beef extract amended autoclaved conditioned medium (ACM) and phosphate buffer (PB). No statistically significant differences in % survival were observed ($P \geq 0.05$).

There were no statistically significant differences observed at any time points between CM, ACM, and PB. Less than 3% of the initial bacterial population survived at the end of survival study.

AI-2 molecules induce gene expression of selected virulence genes

Figure 4 shows the expression of the *yadK* and *hha* genes of VS94 strain in the presence of CM (which contains AI-2 activity) as compared to the ACM. In the presence of CM, the *yadK* and the *hha* genes show a 2.8- and a 1.9-fold respective increase in expression, suggesting that the AI-2 molecules present in CM are able to induce the expression of both these genes. No statistically significant differences in gene expression were observed when CM and ACM were amended with ground beef extracts (Figure 5).

The potential role of autoinducer molecules in modulating microbial persistence, growth, and virulence traits is intriguing. Our understanding about microbial cell signaling molecules, signaling pathways, and how these processes control microbial growth and virulence traits in foods is still very much in its infancy (Smith and others 2004; Pillai and Jesudhasan 2007). There are reports that certain food products contain AI-2 molecules and some can even exhibit AI-2 inhibitory activity (Bjarnsholt and others 2005; Widmer and others 2007). The source of AI-2 could be microbial in origin or foods could contain compounds that can mimic AI-2 autoinducer activity (Teplitski and others 2004).

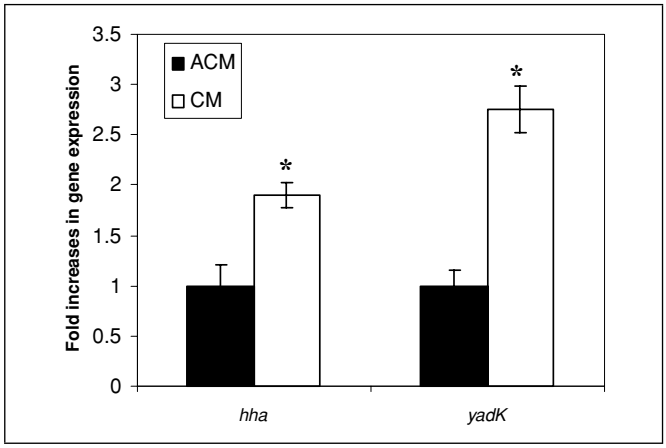

Figure 4—Induced gene expression of *hha* and *yadK* in the presence of AI-2 (conditioned medium [CM]) compared to its absence (autoclaved conditioned medium [ACM]). No statistically significant differences in fold differential gene expression were observed ($P \geq 0.05$).

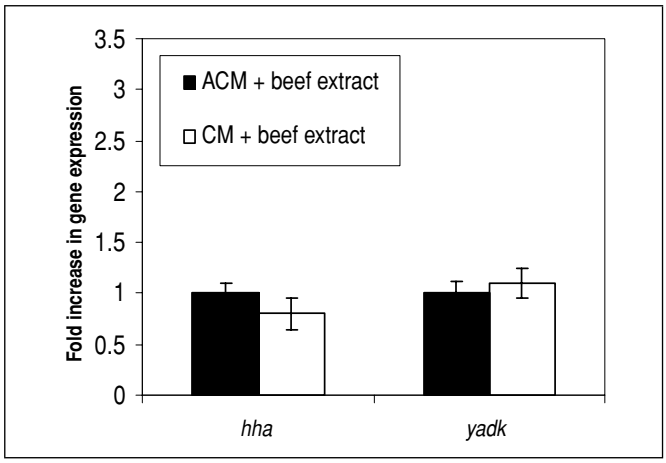

Figure 5—Differential gene expression of *hha* and *yadK* in the presence of beef extract amended AI-2 (conditioned medium [CM]) or beef extract amended autoclaved conditioned medium [ACM]). *Represents statistical significant difference ($P \leq 0.05$) based on paired *t*-test.

Lu and others (2004) have reported that many meat products, including ground beef and poultry meat, are capable of inhibiting AI-2 activity when assayed using *V. harveyi* reporter strain. We have shown in this study (Figure 1) and in a previous study (Lu and others 2004) that ground beef contains compounds that can interfere with the AI-2 sensing in the classical AI-2 sensing *V. harveyi* biosensor assays. In this study, we show that the AI-2 molecules present in conditioned medium were able to give *E. coli* O157:H7 cells a protective effect on survival. However, the protective effect of AI-2 molecules was negated in the presence of ground beef extracts that contained significant amount inhibitory activity.

The putative fimbrial protein gene (*yadK*) and the hemolysin expression-modulating protein gene (*hha*) along with a number of other genes related to virulence, cell division, and small molecule metabolism were shown to be influenced by AI-2. In *E. coli*, synthesis and secretion of the hemolysin are determined by the *hly* operon, while the *hha* gene is involved in the production of the hemolysin regulating protein (Jubete and others 1995). Hemolysin is the cell product that can increase the virulence of *E. coli* strains (Brauner and others 1995). The *yad*K gene is involved in the function such as mobility, secretion, and adhesion (Welch and others 2002). DeLisa and others (2001) had previously identified these 2 genes to be under the control of AI-2 molecules in CM. Microarray analysis has revealed that approximately 79% (56) of the QS regulated genes in *E. coli* were repressed by the presence of synthetic furanones known to inhibit AI-2 activity (Ren and others 2004). In this article, we show that AI-2 inhibitory compounds present in ground beef extracts were also able to negate the influence of AI-2 molecules on gene expression.

Conclusions

There are inhibitors of AI-2 activity in ground beef that limit the survival of *E. coli* O157:H7 and its virulence gene expression. From a practical food safety perspective, these findings imply that certain foods possess compounds that can limit the effect of AI-2 molecules. It is thus important to take cell–cell signaling into account when studying the survival and virulence of foodborne pathogens.

Acknowledgments

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